In Vitro Cell. Dev. Biol.—Plant 39:455–458, September–October 2003
DOI: 10.1079/IVP2003446
© 2003 Society for In Vitro Biology
1054-5476/03 $18.00+0.00

MICROPROPAGATION OF CHINESE REDBUD (*CERCIS YUNNANENSIS*) THROUGH AXILLARY BUD BREAKING AND INDUCTION OF ADVENTITIOUS SHOOTS FROM LEAF PIECES

EUNJU CHEONG[1,2] AND MARGARET R. POOLER[2]*

[1]*Korea Forest Research Institute, 1253 Sanghyodong, Seogwipo, Jeju 697-050, Korea*
[2]*U.S. Department of Agriculture, Agricultural Research Service, U.S. National Arboretum, Floral and Nursery Plants Research Unit, 3501 New York Ave., NE, Washington, DC 20002*

(Received 17 July 2002; accepted 3 January 2003; editor S. Guha-Mukherjee)

Summary

Factors affecting *in vitro* shoot production and regeneration of *Cercis yunnanensis* Hu et Cheng were investigated by comparing various growth regulators and explant types. For optimum shoot production from axillary buds, Murashige and Skoog (MS) media containing 6-benzyladenine, either alone or in combination with a low concentration of thidiazuron, resulted in the greatest number of shoots formed per explant (>3). Explants (2 mm long) containing one axillary bud placed in direct contact with the medium yielded the most shoots per bud (1.6) when grown on growth regulator-free medium. Root formation on 70–80% of shoot explants was accomplished using either indole-3-butyric acid or α-naphthaleneacetic acid in the medium, with significantly more roots formed on explants possessing an apical bud than those without the bud. Direct shoot organogenesis from leaf explants occurred on MS medium containing 10–30 μ*M* thidiazuron, with up to 42% of leaf explants producing shoots.

Key words: organogenesis; propagation; regeneration; tissue culture; thidiazuron.

Introduction

Redbud (*Cercis* spp.) are popular ornamental small trees or shrubs, valued commercially for their showy early spring bloom, heart-shaped glossy leaves, and adaptability to diverse environmental conditions. The genus *Cercis* (Fabaceae) contains 7–13 species or sub-species that occur in North America, Europe, and Asia (Krussmann, 1976; Raulston, 1990; Rehder, 1990). Species range in size from small shrubs to trees, tolerate full sun to shade, and are hardy in USDA Zones 4–9 (Robertson, 1976; Raulston, 1990). Despite the horticultural merits and wide adaptability of the genus, problems with susceptibility to infection by the canker-causing fungus *Botryosphaeria ribis* Gross. et Duggar, as well as difficulty in large-scale production of clonal material, have limited the widespread use of *Cercis* as an ornamental plant. Due to propagation difficulties, studies have been conducted to assess the use of tissue culture for mass propagation of eastern redbud (*C. canadensis* L.) using shoots (Bennett, 1987; Yusnita et al., 1990), cotyledonary nodes (Distabanjong and Geneve, 1997a), and somatic embryos (Distabanjong and Geneve, 1997b). Embryogenesis from immature embryos has been attempted by Trigiano et al. (1988). *In vitro* studies using Chinese redbud, namely *C. chinensis* Bunge, *C. glabra* Pampanini, or *C. yunnanensis* Hu et Cheng, have not been conducted, despite the horticultural merits of these species, including dark green leaves, densely arranged flower buds, and a more compact growth habit than *C. canadensis*; these three taxa may represent a single species (Raulston, 1990). Our objectives in this study were to develop a high-efficiency, multiple shoot production system for *C. yunnanensis* using axillary buds, and to investigate factors leading to adventitious shoot formation from leaf tissue. Development of these systems could be useful in the production of transgenic disease-resistant *Cercis* plants.

Materials and Methods

Plant materials. Seeds from various *Cercis* species were germinated in the greenhouse at the US National Arboretum in Washington, DC in 1999 for use in propagation or disease resistance studies (Pooler and Dix, 2001). Shoot tips from some of these species were harvested, surface-disinfested in 70% ethanol for 1 min, followed by 15 min in 15% commercial bleach with constant stirring. Shoots were rinsed three times with sterile distilled water and cultured on shoot maintenance medium consisting of MS salts and vitamins (Murashige and Skoog, 1962) (pH 6.0) supplemented with 2.5% sucrose, 0.1 μ*M* indole-3-butyric acid (IBA), and 1.0 μ*M* 6-benzyladenine (BA). Media were solidified with 0.15% (w/v) gelrite (Sigma Chemical Co., St. Louis, MO) and 0.35% (w/v) agar (Sigma Chemical Co.). The media were autoclaved for 25 min at 121°C. Growing shoots were subcultured every 4–5 wk. All explants used for mass propagation experiments were transferred from shoot maintenance media to hormone-free MS medium and grown for at least 3 wk prior to experimental manipulations. Cultures were maintained at approximately 23°C under cool white fluorescent light with a 16 h photoperiod of 40 $\mu mol\, m^{-2}\, s^{-1}$ light intensity.

Preliminary experiments identified one accession, NA65636, received in 1993 from a 1988 Index Seminum as *C. yunnannensis* Hu et Cheng from Hortus Botanicus Kunminensis, Academiae Sinicae, Kunming Yunnan, China, that proliferated well in culture and also was able to produce adventitious shoots from leaf pieces when cultured on 12.0 μ*M* thidiazuron (TDZ). This accession was used for subsequent experiments to test the effects of various treatments on shoot production and regeneration.

*Author to whom correspondence should be addressed: Email poolerm@ars.usda.gov

Effects of cytokinins on shoot multiplication. Five cytokinins were tested to determine which ones were most effective in stimulating bud flush and multiple shoot development. Basal media containing MS salts and vitamins (pH 5.8) with 3.0% sucrose and solidified with 0.1% gelrite (w/v) and 0.35% agar (w/v) was supplemented with 5.0 μM of either BA, kinetin, 2-isopentenyladenine (2ip), TDZ, or zeatin. An explant consisted of a ~10 mm shoot with one axillary bud. Two replications of five explants each were cultured for each treatment. Explants were cultured in 100 ml (66 mm high) glass baby food jars. Shoot number and length were recorded after 4 wk in culture. Based on the results of the cytokinin test, BA and TDZ were further tested at 1.0, 3.0, 5.0, and 10.0 μM using the same explant type and basal media described above. Finally, since shoot length was maximum at 3 μM BA and shoot number was greatest at 10 μM BA, possible synergistic effects of BA and TDZ were tested by using 3.0 or 10.0 μM BA in combination with 0, 1.0, or 2.0 μM TDZ. Explants were cultured in 30 × 150 mm glass test tubes. The environmental conditions were the same as described previously.

Effects of explant type on shoot multiplication. The effects of explant size and number of buds on shoot induction were also investigated. The following four explant types were tested: (a) short stem (2 mm) with one axillary bud; (b) long stem (10 mm) with one axillary bud; (c) long stem with two axillary buds; (d) long stem with three axillary buds. Explants were placed on media containing MS salts and vitamins (pH 5.8), supplemented with 3% sucrose and 3 μM BA and solidified with 0.1% gelrite and 0.35% agar. Buds of explant type (a) were placed directly on the medium, while buds of the other three explant types did not contact the media. Explants were cultivated in 30 × 150 mm glass test tubes. The environmental conditions were the same as described above.

Root development of propagated shoots in vitro. Two experiments were conducted to optimize adventitious root formation from shoots. The effects of different auxins and the presence of the apical bud on rooting were tested by comparing rooting efficiency between explants with and without apical buds, using IBA or α-naphthaleneacetic acid (NAA). Stem segments 2 cm long were harvested from actively growing cultures on growth regulator-free MS medium. One explant type contained the tip of the stem, and included one apical and one axillary bud; the other explant type included two axillary buds and no apical bud. Shoots were placed on media containing MS salts and vitamins (pH 5.8), 3% sucrose, 0.1% gelrite, 0.35% agar, and 1.0 or 5.0 μM IBA or NAA. For each treatment, five explants were cultured in a Magenta® GA7 vessel, with two replications. Data were recorded after 4 wk in culture.

Adventitious shoot induction from leaf tissue. Leaves from the upper one-third of actively growing shoots cultured on shoot maintenance medium were removed and squeezed gently with non-traumatic surgical forceps to wound the tissue. Leaves were then placed on media in 100 × 15 mm polystyrene Petri plates containing MS salts and vitamins (pH 6.0) with 2.5% sucrose, solidified with 0.15% gelrite and 0.35% agar (w/v), and supplemented with 1.0 μM IBA and 1, 10, or 30 μM TDZ. Half the leaves were placed with abaxial side in contact with the media, and half were placed with adaxial side in contact with the media. Cultures were kept in the dark for 0, 4, or 8 wk prior to exposing them to light. Five leaves were cultured on each plate, and two plates were cultured for each TDZ/dark treatment/leaf placement combination. After cultures were removed from the dark (or at 4 wk for the 0 dark treatment), leaves were transferred to medium containing 0.05 μM IBA and 8.9 μM BA, with no TDZ. Number of leaves with shoots or roots, and number of shoots per leaf were recorded 12 wk after transferring to the TDZ-free media.

Experimental design and statistics. Treatments in all experiments were arranged in a completely randomized design. Analysis of variance, treatment means, and standard errors were determined using Statistix® 7 for Windows software (Analytical Software Co., La Jolla, CA). For those treatments where ANOVA showed significant effects ($P < 0.05$), least significant difference (LSD) was used to compare means.

Results and Discussion

Effects of cytokinins on shoot multiplication. At a fixed concentration of 5 μM, the type of cytokinin used had a significant effect on both the number of shoots induced ($P < 0.001$) and the average length of the shoots ($P = 0.03$) (Table 1; Fig. 1). Explants grown on media containing TDZ produced significantly more shoots than explants from the other treatments, but were often hyperhydric and distorted. The TDZ treatment resulted in multiple shoot formation from the axillary bud as well as multiple shoots from callus at the base of the explant. Explants grown on media containing BA or zeatin produced significantly more shoots and longer shoots than explants grown on kinetin or 2iP, and also formed multiple shoots from the axillary bud and the basal callus, although to a lesser extent than explants grown on TDZ. Our results are consistent with those of previous reports on the negative effects of TDZ on shoot quality (Malik and Saxena, 1992; Heutleman and Preece, 1993; Lu, 1993), and the superiority of BA over 2iP in shoot production (Bennett, 1987).

TABLE 1

EFFECTS OF VARIOUS CYTOKININS ON SHOOT FORMATION IN *CERCIS YUNNANENSIS*

Cytokinin (5 μM)	Mean no. of shoots formed per explant	Mean length of shoots (mm)
TDZ	2.5 a	24 b
BA	1.4 b	30 a
Zeatin	1.4 b	28 ab
Kinetin	1.0 c	21 c
2ip	1.0 c	23 bc

Different *letters* indicate mean separation within *columns* using LSD ($P < 0.05$).

FIG. 1. Effects of various cytokinins on shoot elongation in *Cercis yunnanensis*.

Further tests using varying concentrations of BA and TDZ indicated that increasing the concentration of BA had little effect on the number of shoots formed (mean 1.0 shoot per explant), whereas concentrations of TDZ at and above 3 μM stimulated more shoot development (>2 shoots per explant) than at 1 μM. However, high concentrations of BA (10 μM) and all levels of TDZ resulted in shorter shoots, increased shoot tip necrosis, and red pigmentation in the leaves. Our investigation confirms the report by Yusnita et al. (1990) that medium containing TDZ promoted the formation of numerous axillary shoots, although these shoots were distorted and usually failed to elongate.

We also tested BA and TDZ in combination to determine if multiple elongated shoots could be produced from a synergistic

effect of the two growth regulators. The addition of TDZ to media containing BA resulted in more and shorter shoots per explant (Table 2), although there were few significant differences among the various treatments. Previous studies with *C. canadensis* (Distabanjong and Geneve, 1997a) found a synergistic effect of the two growth regulators, although results from our study suggest an additive effect, at best. Transferring newly induced shoots from media containing TDZ and/or BA to media containing only BA could also optimize both shoot number and shoot elongation (Nielsen et al., 1995).

Effects of explant type on shoot multiplication. The type of explant used also had a significant effect on the number of shoots produced ($P < 0.01$, Table 3). Explants with one axillary bud produced a mean of at least one shoot for each axillary bud; however, explants with two or three axillary buds did not produce a shoot for each bud; rather, apical dominance resulted in only one bud producing a shoot in most cases. Therefore, in terms of the number of shoots produced per bud cultured, fewer axillary buds on the explant appear to be most efficient. Explant type did not have a significant effect on the length of shoots produced. All explants placed on hormone-free media produced only one shoot per explant, regardless of explant type, thus confirming the role of BA in stimulating adventitious bud development. In addition to shoot induction from the axillary bud, some of the explants, most notably explants of type (a), produced shoots from the base of the explant. In our *Cercis* system, the increased contact of the shorter explant with the medium likely played a role in its superior ability to produce multiple shoots from axillary buds as well as from the base of the explant.

Root development of propagated shoots in vitro. The development of adventitious roots from shoots was dependent on the explant type, with no significant effect of various types and concentrations of auxins in the medium. Explants that contained both an apical bud and an axillary bud developed adventitious roots significantly more frequently than explants containing two axillary buds and no apical bud on growth regulator-free media (79% vs. 48%) and on media containing 5.0 μ*M* NAA (75% vs. 34%), most likely due to the presence of endogenous auxins synthesized in the apical bud. In addition, explants without apical buds growing on media containing 5.0 μ*M* NAA produced large amounts of callus at the base of the shoot. Otherwise, there was no effect of auxin type or concentration on rooting *in vitro*.

Adventitious shoot induction from leaf tissue. Media containing 30 μ*M* TDZ was the most effective treatment in promoting formation of adventitious shoots from leaf tissue (Table 4), although the concentration of TDZ did not affect the average number of shoots per regenerating leaf. No shoot and significantly more roots were induced from leaves grown on media with low (1.0 μ*M*) TDZ concentrations. Future experiments with TDZ concentrations above 30 μ*M* are necessary to establish an optimum level for induction of shoots. The duration of dark exposure had no significant effect on the number of leaves with shoots or the average number of shoots per leaf; however, there were significantly more roots produced with increasing duration of dark treatment. The placement of the leaf on the media (abaxial surface up or down) had no effect on regeneration in this experiment. There appeared to be variation between leaves on a plate, as some leaves produced only callus, others produced a single, well-formed shoot, while others produced multiple smaller, weaker shoots (Fig. 2).

TABLE 2

MEAN SHOOT LENGTH AND MEAN NUMBER OF SHOOTS PRODUCED PER EXPLANT ON MEDIA WITH VARIOUS COMBINATIONS OF BA AND TDZ

BA (μ*M*)	TDZ (μ*M*)	Mean no. of shoots formed per explant	Mean length of shoots (mm)
3	0	1.2 a	32.7 a
3	1	2.5 b	23.8 ab
3	2	3.7 b	17.3 b
10	0	2.5 b	22.0 b
10	1	3.6 b	16.2 b
10	2	3.8 b	18.8 b

Different *letters* indicate mean separation within *columns* using LSD ($P < 0.05$).

TABLE 3

EFFECTS OF EXPLANT TYPE ON SHOOT FORMATION IN *CERCIS YUNNANENSIS* [a]

Explant type	Mean no. shoots produced[z]	Mean shoot length (mm)[y]	% Explants with multiple shoots
a: short stem, one axillary bud	1.6 b	29.3	46
b: long stem, one axillary bud	1.0 c	32.7	0
c: long stem, two axillary buds	1.6 b	23.7	5
d: long stem, three axillary buds	2.3 a	22.8	15

[z] Different *letters* indicate mean separation within *columns* using LSD ($P < 0.05$).

[y] ANOVA indicated nonsignificant *P* value for differences in shoot length among treatments.

TABLE 4

EFFECTS OF TDZ CONCENTRATION AND DURATION OF DARK TREATMENT ON REGENERATION OF *CERCIS YUNNANENSIS* FROM LEAF TISSUE

Treatment	Percentage of leaves producing shoots[z]	Mean no. of shoots per leaf[z,y]	Percentage of leaves producing roots[z]
TDZ (μ*M*)			
1	0 a	(0)	40 a
10	18 b	2.8 a	8 b
30	42 c	2.8 a	2 b
Weeks in dark			
0	8 a	2.0 a	8 a
4	16 ab	3.7 a	22 a
8	36 b	2.7 a	20 a

Means for TDZ concentrations are for all dark treatments combined, and means for dark treatments are for all TDZ concentrations combined.

[z] Different *letters* indicate mean separation within *columns* using LSD ($P < 0.05$).

[y] Mean is derived only from leaves that produced shoots.

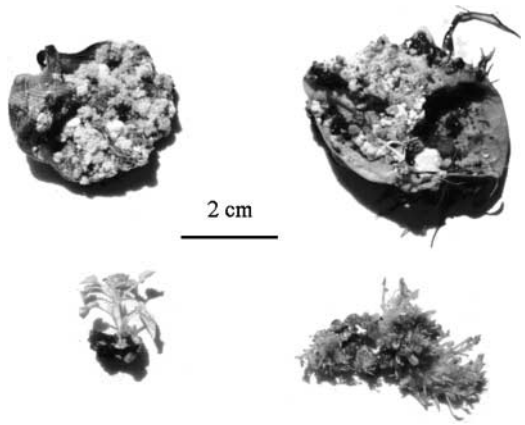

FIG. 2. Regeneration of *Cercis yunnanensis* from leaf pieces, showing (clockwise from *top left*): callus formation on leaf; adventitious root formation from leaf; clump of adventitious shoots excised from leaf explant; single, well-developed adventitious shoot excised from leaf explant.

Although somatic embryogenesis in redbud can be accomplished (Trigiano et al., 1988; Geneve, 1991; Trigiano et al., 1995), regeneration of *Cercis* directly from leaf tissue has not been reported. Our preliminary screening of *Cercis* species for ability to regenerate in culture revealed a strong effect of genotype, as only *C. yunnanensis* NA65636 and one selection of *C. glabra* (NA69817) produced regenerating shoots. Interestingly, *C. yunnanensis* and *C. glabra*, both native to China, are thought to be closely related, if not identical, to *C. chinensis* (Raulston, 1990).

For optimum *in vitro* propagation of this clone of *C. yunnanensis*, we recommend culturing explants of type (a) on MS media with 3 μ*M* BA and 1 μ*M* TDZ. Rooting of resulting shoots with apical buds can be accomplished using 1.0–5.0 μ*M* IBA. Using these methods, an average of two rooted plantlets can be produced per bud in 3 mo. We hope to use this regeneration and shoot multiplication system to introduce novel genes for disease resistance into cultivated *Cercis* species.

REFERENCES

Bennett, L. Tissue culturing redbud. Am. Nurseryman 166: 85–87, 90–91; 1987.

Distabanjong, K.; Geneve, R. L. Multiple shoot formation from cotyledonary node segments of eastern redbud. Plant Cell Tiss. Organ Cult. 47:247–254; 1997a.

Distabanjong, K.; Geneve, R. L. Multiple shoot formation from normal and malformed somatic embryo explants of eastern redbud (*Cercis canadensis* L.). Plant Cell Rep. 16:334–338; 1997b.

Geneve, R. L. Eastern redbud (*Cercis canadensis* L.) and judas tree (*Cercis siliquastrum* L.). In: Bajaj, Y. P. S., ed. Biotechnology in agriculture and forestry, vol. 16, Trees. Berlin: Springer-Verlag; 1991:142–151.

Heutleman, C. A.; Preece, J. E. Thidiazuron; a potent cytokinin for woody plant tissue culture. Plant Cell Tiss. Organ Cult. 33:105–119; 1993.

Krussmann, G. Manual of cultivated broad-leaved trees and shrubs, vol. I, A–D. Beaverton, OR: Timber Press; 1976.

Lu, C. The use of thidiazuron in tissue culture. In Vitro Cell. Dev. Biol. 29P:92–96; 1993.

Malik, K. A.; Saxena, P. K. Thidiazuron induces high-frequency shoot regeneration in intact seedlings of pea (*Pisum sativum*), chickpea (*Cicer arietinum*) and lentil (*Lens culinaris*). Australian J. Plant Physiol. 19:731–740; 1992.

Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–497; 1962.

Nielsen, J. M.; Hansen, J.; Brandt, K. Synergism of thidiazuron and benzyladenine in axillary shoot formation depends on sequence of application in *Miscanthus* × *ogiformis* 'Giganteus'. Plant Cell Tiss. Organ Cult. 41:165–170; 1995.

Pooler, M. R.; Dix, R. L. Screening of *Cercis* (redbud) taxa for ability to root from cuttings. J. Environ. Hort. 19:137–139; 2001.

Raulston, J. C. Redbud. Am. Nurseryman 171(5):39–51; 1990.

Rehder, A. Manual of cultivated trees and shrubs, 2nd edn. Portland, OR: Dioscorides Press; 1990.

Robertson, K. R. *Cercis*: the redbuds. Arnoldia 36(2):37–49; 1976.

Trigiano, R. N.; Beaty, R. M.; Graham, E. T. Somatic embryogenesis from immature embryos of redbud (*Cercis canadensis*). Plant Cell Rep. 7:148–150; 1988.

Trigiano, R. N.; Geneve, R. L.; Buckley, L. G. Somatic embryogenesis in eastern redbud (*Cercis canadensis*). In: Jain, S.; Gupta, P.; Newton, R., eds. Somatic embryogenesis in woody plants, vol. 2. Dordrecht: Kluwer Academic Publishers; 1995:471–482.

Yusnita, S.; Geneve, R. L.; Kester, S. T. Micropropagation of white flowering eastern redbud (*Cercis canadensis* var. *alba* L.). J. Environ. Hort. 8:177–179; 1990.